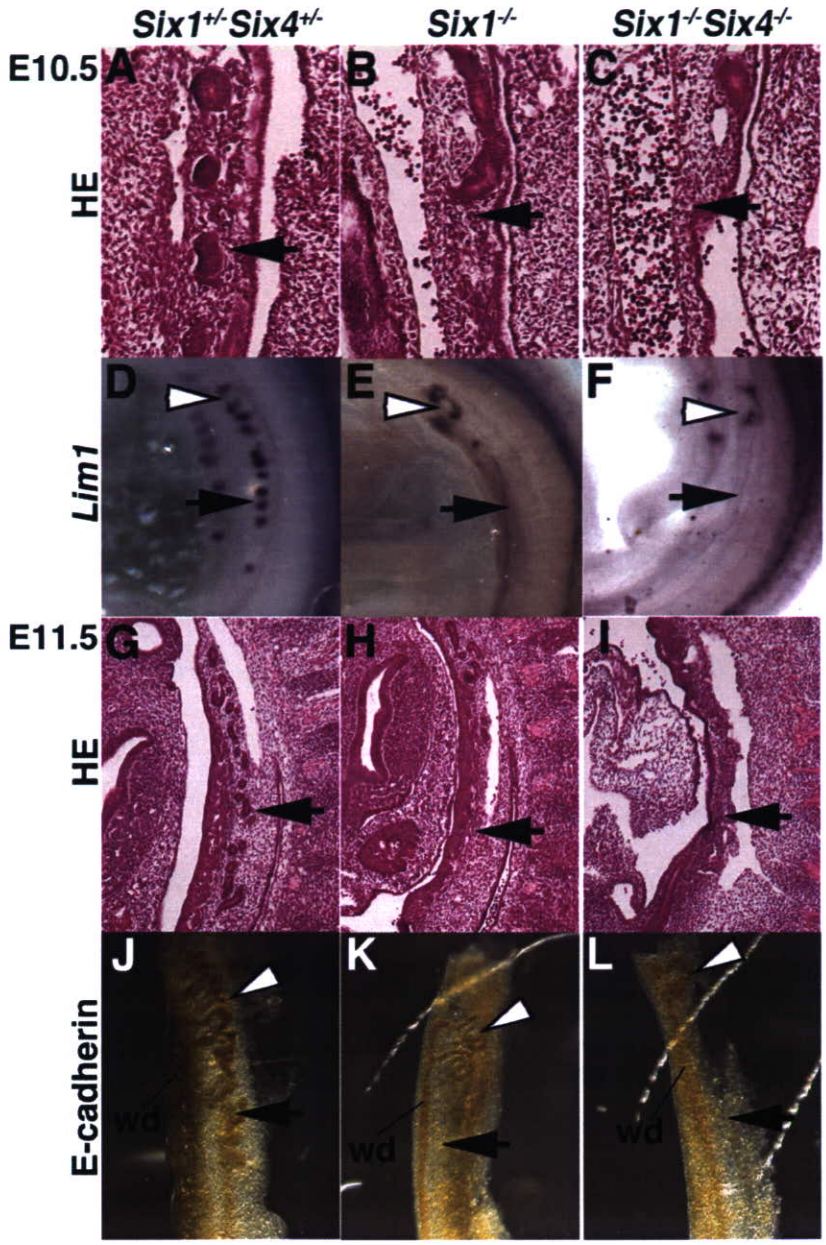

Fig. 5. Caudal mesonephric tubules are not formed in the *Six1*- and *Six1/Six4*-deficient embryos. (A–C) HE staining of the sagittal sections of the mesonephric region of the E10.5 *Six1/Six4*-heterozygous (A), *Six1*-deficient (B), and *Six1/Six4*-deficient (C) embryos. Several mesonephric tubules (arrow) were observed in the *Six1/Six4*-heterozygous embryos (A) but they were fewer in the *Six1*- (B) and *Six1/Six4*-deficient embryos (C). (D–F) Whole-mount in situ hybridization of *Lim1* in the mesonephros of the E10.5 *Six1/Six4*-heterozygous (D), *Six1*-deficient (E), and *Six1/Six4*-deficient (F) embryos. *Lim1*-positive mesonephric tubules were observed lined in the craniocaudal direction in the *Six1/Six4*-heterozygous embryos (D), while *Lim1*-positive caudal mesonephric tubules (black arrow) were not observed in the *Six1*- (E) and *Six1/Six4*-deficient (F) embryos. (G–I) HE staining of the sagittal sections of the mesonephric region of the E11.5 *Six1/Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1/Six4*-deficient (I) embryos. Many mesonephric tubules were observed in the *Six1/Six4*-heterozygous embryos (G), while few were observed in the *Six1*- (H) and *Six1/Six4*-deficient embryos (I). (J–L) Whole-mount immunostaining of E-cadherin, which is expressed in the mesonephric tubules and the Wolffian duct, in the mesonephros of the E11.5 *Six1/Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1/Six4*-deficient (L) embryos. Many mesonephric tubules were observed lined in the craniocaudal direction in the *Six1/Six4*-heterozygous embryos (J), while caudal mesonephric tubules were not observed in the *Six1*- (K) and *Six1/Six4*-deficient embryos (L). The white arrowhead indicates the cranial mesonephric tubules (wd, Wolffian duct).

Six1-deficient mice, and *Six4* may play a minimal role in the expression of this gene. This differential regulation could result either from the difference in the amount of the two corresponding gene products in the tissue or from the binding specificity of the DNA-binding domains to the target sequences. Previously, we reported that the promoter region of the *sodium-potassium-chloride cotransporter 1*(*Scl1 2a2*) gene is a common target of Six1 and Six4,

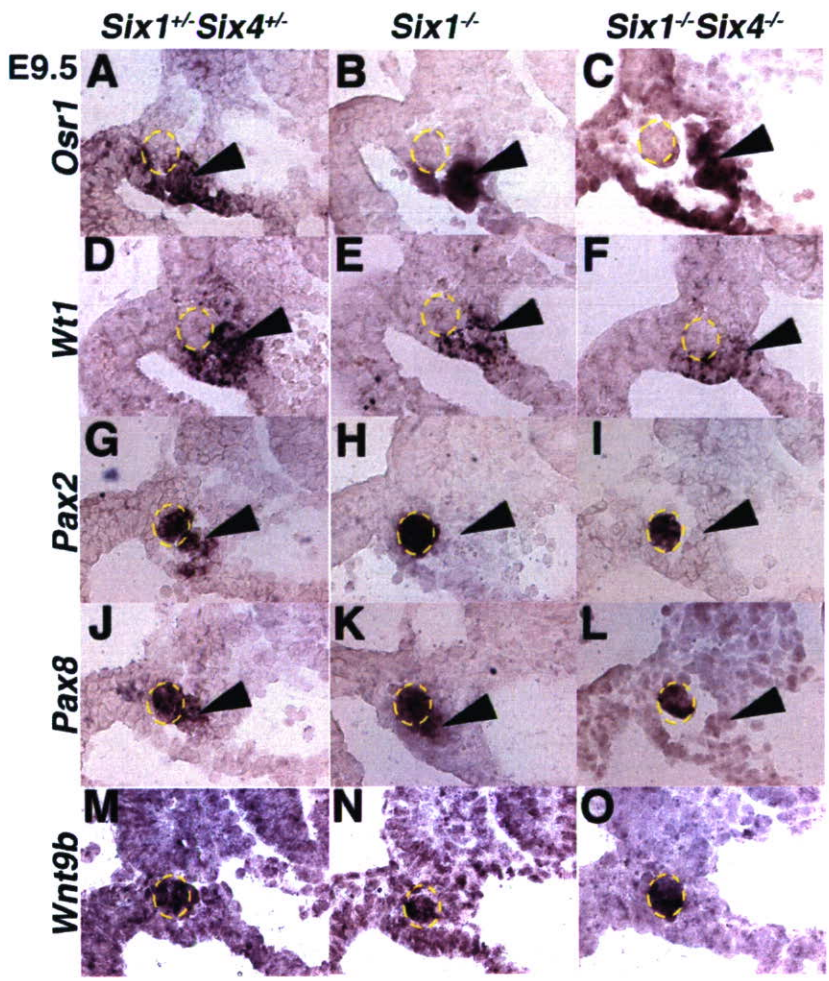

Fig. 6. *Pax2* expression is absent in the nephrogenic cord in *Six1*-deficient embryos. (A–C) In situ hybridization of *Osr1* in the mesonephric region of the E9.5 *Six1*/*Six4*-heterozygous (A), *Six1*-deficient (B), and *Six1*/*Six4*-deficient (C) embryos. *Osr1* expression in the nephrogenic cord (arrow) remained intact in the *Six1*- (B) and *Six1*/*Six4*-deficient embryos (C). (D–F) *Wt1* expression in the mesonephric region of the *Six1*/*Six4*-heterozygous (D), *Six1*-deficient (E), and *Six1*/*Six4*-deficient (F) embryos at E9.5. *Wt1* expression in the nephrogenic cord (D) remained intact in the *Six1*- (E) and *Six1*/*Six4*-deficient (F) embryos. (G–I) *Pax2* expression in the mesonephric region of the E9.5 *Six1*/*Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1*/*Six4*-deficient (I) embryos. *Pax2* expression in the nephrogenic cord was absent in the *Six1*- (H) and *Six1*/*Six4*-deficient (I) embryos. (J–L) *Pax8* expression in the mesonephric region of the *Six1*/*Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1*/*Six4*-deficient (L) embryos at E9.5. *Pax8* expression in the nephrogenic cord is absent in the *Six1*/*Six4*-deficient embryos (L) but not in the *Six1*-deficient embryos (K). (M–O) *Wnt9b* expression in the mesonephric region of the E9.5 *Six1*/*Six4*-heterozygous (M), *Six1*-deficient (N), and *Six1*/*Six4*-deficient (O) embryos. *Wnt9b* expression in the Wolffian duct (yellow circle) was unaffected in the mutant embryos.

and that this region contains multiple Six1-binding sites and one common binding site for Six1 and Six4 (Ando et al., 2005). A similar mechanism may also underlie the differential regulation of common target genes during kidney development.

The *Six* gene family has a consensus binding site, and Six1 is reported to bind to the first intron of *Gdnf* (Li et al., 2003) – a key attractant for ureteric budding. Further, Pax2 binds to the 5'-untranslated region of *Gdnf* exon 1 (Brophy et al., 2001). Six2 also binds to the *Gdnf* promoter region and activates *Gdnf* expression (Brodbeck et al., 2004). However, *Six2* expression is absent in *Six1*-deficient mouse embryos (data not shown; Xu et al., 2003). These results are consistent with the findings that both the *Six1*- and *Pax2*-deficient mice show incomplete ureteric-bud invasion into the mesenchyme (Brophy et al., 2001; Li et al., 2003). Though the mechanism underlying the direct binding of Six4 to the promoters remains to be elucidated, *Six1* and *Six4* may regulate *Gdnf* expression in two ways – directly or indirectly. The former is executed by binding to the *Gdnf* promoter and the latter, via *Pax2* and *Pax8*. Thus, inactivation of *Six1* and *Six4* should lead to a complete absence of *Gdnf*, a critical factor for ureteric bud formation. This reveals a redundancy in the *Six-Pax-Gdnf* pathway. *Eya1* is a cofactor for *Six1*-dependent transcriptional activation, and the loss of *Eya1* in mice leads to a metanephric phenotype similar to that of *Six1*/*Six4*-deficient mice and more severe than that of

the *Six1*-deficient mice; *Pax2* and *Gdnf* expression is completely absent in this phenotype (Xu et al., 1999; Nica et al., 2006). Hence, it is possible that both Six1 and Six4 cooperate with Eya1, thus regulating *Pax2/Pax8* and *Gdnf* expression.

Mesonephric tubules were absent in both the *Six1*- and *Six1/Six4*-deficient mice. Although several researchers have reported metanephric abnormalities in *Six1*-null mice (Laclef et al., 2003a; Li et al., 2003; Xu et al., 2003), this is the first report of mesonephric phenotypes in these mice. *Pax2* was absent in the *Six1*- and *Six1/Six4*-deficient mice, while *Pax8* remained undetected only in the *Six1/Six4*-deficient mice. This resembled a cascade similar to that occurring during metanephros formation. However, the absence of *Six4* did not exacerbate the defects observed in *Six1* deficiency; this suggests that a reduction in *Pax8* expression does not contribute to the lack of mesonephric tubules and that *Pax2* expression may play a major role in this process. Consistent with this finding, Torres et al. (1995) reported that mesonephric-tubule formation is impaired in *Pax2*-deficient mice. Thus, *Six1*-dependent *Pax2* activation in the nephrogenic cord (a mesenchymal tissue) could be a prerequisite for mesonephric-tubule formation, which involves a mesenchymal-to-epithelial transition induced by signals, including Wnt9b from the Wolffian duct (Carroll et al., 2005).

Pax2 and *Pax8* are expressed in the intermediate mesoderm, and these two genes must play a major role in directing the intermediate mesoderm toward nephrogenic lineage because mice deficient in both these genes lack Wolffian-duct formation (Bouchard et al., 2002). In contrast, in *Six1/Six4*-null mice, direction toward kidney lineage occurs, although *Pax2* and *Pax8* expression is abolished. This apparent inconsistency can be explained by studying the expression domains of these genes. *Six1* and *Six4* are expressed in the nephrogenic mesenchyme (nephrogenic cord and metanephric mesenchyme) but not in the Wolffian duct, while *Pax2* and *Pax8* are expressed in each of these intermediate mesoderm-derived components. In the absence of *Six1* and *Six4*, *Pax2* and *Pax8* expression is reduced in the nephrogenic mesenchyme but not in the Wolffian duct (Fig. 6T, L). Therefore, *Six1/Six4*-null mice tend to exhibit mesenchyme-specific lowering of *Pax2* and *Pax8* expression; thus, they exhibit a milder phenotype than conventional *Pax2/Pax8*-deficient mice. In the mesenchyme, *Six1* and *Six4* are located upstream of *Pax2* and *Pax8*, as mentioned in this paper. This cascade does not exist in the Wolffian duct. Instead, *Pax2/Pax8*-dependent *Gata3* activation is reported to regulate migration of the Wolffian duct toward the cloaca. In *Gata3*-null mice, mesenchymal migration is maintained, and *Pax2* expression in the mesenchyme (and possibly the expression of its upstream regulators *Six1* and *Six4*) is not affected (Grote et al., 2006). Thus, the *Six-Pax* cascade in the mesenchyme and the *Pax-Gata3* pathway in the Wolffian duct may operate independently.

Our data place *Six1* and *Six4* upstream of *Pax2* and *Pax8* in the mesenchymal tissues in both the metanephros and mesonephros. In addition, our data further elaborates on the findings of Xu et al., revealing that *Six1* is located upstream of *Pax2* during metanephric development (Xu et al., 2003). This *Six-Pax* cascade is distinct from the situation in *Drosophila*, in which *sine oculis* (*Six* homolog) is located downstream of *eyeless* (*Pax* homolog). However, the *Six-Pax* cascade is conserved during mammalian muscle development, where *Six1* and *Six4* are located upstream of *Pax3* and *Met* and regulate myogenic migration in somites (Grifone et al., 2005). Then how are *Six1* and *Six4* regulated in the nephrogenic mesenchyme? A recent study predicted the TCF4- and Gli-binding sites in the promoters of *Six1* and *Six4* by using computational methods, suggesting that Wnt and hedgehog may regulate the *Six* genes (Hallikas et al., 2006). In fact, *Six1* expression in the limb buds is reduced in *Shh* mutants (Bonnin et al., 2005). In the metanephros, *Shh* deficiency leads to a reduction in *Pax2* and *Sall1* genes downstream of *Six1* (Hu et al., 2006). Thus, an analysis using mutant mice that lack the candidate soluble factors may further reveal the details of the *Six-Pax* cascade that controls kidney formation.

Finally, *Six1* mutation is reported to be associated with the branchio-oto-renal syndrome, which leads to kidney or urinary tract malformation (Ruf et al., 2004). In addition, a deletion at 14q22–23 that overlaps the *SIX1* and *SIX4* loci causes multiple abnormalities, including renal hypoplasia (Bennett et al., 1991). Thus, an analysis of *Six1/Six4*-deficient mice may be useful for elucidating the mechanism underlying these diseases.

4. Experimental procedures

4.1. Generating mutant mice and confirming their genotype

The procedure for generating *Six1*- and *Six1/Six4*-deficient mice has been previously described (Ozaki et al., 2004; Konishi et al., 2006). *Six1/Six4*-deficient mice carry a targeted in-frame fusion of the *EGFP* gene into the first coding exon of the *Six1* gene and that of the *LacZ* gene into the first coding exon of the *Six4* gene. Mice and embryos from subsequent generations were genotyped by PCR. The *Six1* mutant allele was detected using the primers WtmSix1F (5′-GCGCCCGGGCC CGTGCGCCCC-3′) and KOmSix1R (5′-TGCCCCAGGATGTTGCC GTCC-3′), and the wild-type allele was detected using the primers WtmSix1F and WtmSix1R (5′-GCTTTCAGCCACAGCTGCTGC-3′). The length of the PCR products was 323 and 470 bp in the wild-type and mutant alleles, respectively. The *Six4* mutant allele was detected using the primers WtmSix4F (5′-ACATCAAGCAGGAGAATGGGATGG-3′) and KOmSix4R (5′-CCGTAATGGGATAGGTTACGTTGG-3′), and the wild-type allele was detected using the primers WtmSix4F and WtmSix4R (5′-AGAAGTTCCGAGTGGAGTTGTACC-3′). The length of the PCR products was 212 and 445 bp in the wild-type and mutant alleles, respectively.

4.2. Histological examination

The embryos were fixed in 10% formalin in phosphate-buffered saline (PBS). The dehydrated specimens were embedded in paraffin wax and subsequently cut into 6-μm thick serial sections, de-waxed, and then stained with HE.

4.3. RNA in situ hybridization

The embryos were fixed by incubating them at 4 °C overnight in 10% formalin in PBS. After washing the samples with PBS containing 30% sucrose, they were embedded in OCT compound (Tissue-Tek), frozen, and cut into 10-μm thick serial cryosections. In situ hybridization was performed using digoxigenin-labeled antisense riboprobes, as described previously (Nishinakamura et al., 2001). We used the TSA-amplification kit (PerkinElmer) when required. For whole-mount in situ hybridization, the dissected urogenital tissues were fixed by incubating them at 4 °C overnight in 10% formalin in PBS; they were then dehydrated using a methanol series. Prior to hybridization, the specimens were treated with 20 μg/ml proteinase K (Roche) for 15 min, followed by hybridization with a labeled RNA probe at 68 °C overnight (1 μg/ml). Post hybridization, the specimens were washed twice with 0.1% CHAPS/2 × SSC at 68 °C for 20 min each time; they were then incubated with RNase A (20 μg/ml; 2 × SSC; 37 °C; 30 min) and washed twice with 0.1% CHAPS/0.2 × SSC at 68 °C for 20 min each time. Further, the specimens were blocked with PBS containing 10% sheep serum and 0.1% Triton X-100 and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000 in PBT, Roche) overnight at 4 °C. The samples were developed by using NBT and BCIP. We used the cDNA of the following genes as in situ hybridization probes: *Pax2*, *WT1*, *Sall1*, *Gdnf* (Nishinakamura et al., 2001), and *Wnt9b* (a kind gift from A.P. McMahon). The cDNA for other probes was isolated by PCR, subcloned into pBluescript KS(-), and sequenced. None of the sense probes produced signals.

4.4. Whole-mount immunohistochemistry

The dissected urogenital tissues were fixed in 10% formalin in PBS and treated with 0.3% H_2O_2 in methanol for 60 min. After washing the specimens with PBT (PBS with 1% Triton X-100), they were incubated at 4 °C for 2 h in a blocking solution containing 1% skim milk and 1% normal goat serum in PBT. The specimens were then incubated at 4 °C for 16 h with a mouse primary antibody against E-cadherin (1:1000 in PBT, Becton Dickinson). They were then washed with PBT and incubated at 4 °C for 16 h with a peroxidase-conjugated goat secondary antibody against rabbit IgG (1:500 in PBT, KPL). The samples were developed by incubating them for 30 min with 0.2 mg/ml DAB and 0.01% H_2O_2 in PBS. The specimens were fixed in 10% formalin in PBS and cleaned with 75% glycerol for microscopic observation.

4.5. TUNEL analysis

The cryosections were fixed in 1% paraformaldehyde in PBS and permeabilized with 30% acetic acid in ethanol for 5 min at −30 °C. TUNEL analysis was performed by using the Apoptag In Situ Cell Death Detection Kit (Chemicon). In brief, fragmented DNA in apoptotic cells was end-labeled with digoxigenin, and the labeled DNA was detected using a peroxidase-conjugated anti-digoxigenin antibody, stained using a metal-enhanced DAB kit (Pierce), and counterstained with hematoxylin.

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Stem cells in the embryonic kidney

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The mammalian kidney, the metanephros, is formed by a reciprocally inductive interaction between two precursor tissues, the metanephric mesenchyme and the ureteric bud. The ureteric bud induces the metanephric mesenchyme to differentiate into the epithelia of glomeruli and renal tubules. Multipotent renal progenitors that form colonies upon Wnt4 stimulation and strongly express *Sall1* exist in the metanephric mesenchyme; these cells can partially reconstitute a three-dimensional structure in an organ culture setting. *Six2* maintains this mesenchymal progenitor population by opposing *Wnt4*-mediated epithelialization. Upon epithelial tube formation, Notch2 is required for the differentiation of proximal nephron structures (podocyte and proximal tubules). In addition, the induction methods of the intermediate mesoderm, the precursor of the metanephric mesenchyme, begin to be elucidated. If derivation of metanephric mesenchyme becomes possible, we will be closer to the generation and manipulation of multiple cell lineages in the kidney.

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STRATEGY TOWARD KIDNEY RECONSTITUTION USING PROGENITOR CELLS

Stem cells are defined by two criteria: self-renewal and multipotency. Few reports in the kidney field have addressed both of these criteria at a clonal level, so it is better to use the term 'progenitor' rather than 'stem cells.' In this review, renal progenitors in the embryonic kidney, not those in the adult kidney, from the viewpoint of developmental biology and stem/progenitor cell biology will be discussed. To generate multiple cell lineages for kidney regeneration, the identification of renal progenitors is a prerequisite. Furthermore, there exist three obstacles to be overcome: (1) derivation of the renal progenitors; (2) expansion of the renal progenitors; and (3) control of lineage commitment of the renal progenitors toward differentiated cell types. Recent studies are beginning to elucidate the molecular bases of these processes and will hopefully be able to get closer to the goal of kidney regeneration.

METANEPHRIC MESENCHYME AS A PROGENITOR CELL POPULATION

The mammalian kidney, the metanephros, is formed by a reciprocally inductive interaction between two precursor tissues, the metanephric mesenchyme and the ureteric bud. Upon induction by the ureteric bud, the metanephric mesenchyme differentiates into the epithelia of glomeruli and renal tubules. Mesenchymal cells sequentially form condensates, renal vesicles, comma (C)- and S-shaped bodies, and terminal epithelia of glomeruli and renal tubules. A previous report retrospectively suggested the presence of multipotent cells in embryonic kidneys, demonstrating that cells in several portions of the nephron were derived from a single stem cell, using LacZ gene transduction with a retrovirus into a single cell of the mesenchyme.[1] However, no one has isolated renal progenitor cells with multilineage differentiation potential from the embryonic kidney or has examined their differentiation mechanisms in a single cell culture. There has been a lack of assay systems that specifically identify renal progenitors, as in the cases of the neurosphere method for neural stem cells and the colony assay for hematopoietic progenitors.

RENAL PROGENITORS IN THE *SALL1*-HIGH POPULATION

Targeted disruption of *Wnt4* results in kidney agenesis and impairs mesenchymal-to-epithelial transition,[2] and co-culture of the mesenchyme with NIH3T3 cells expressing

Wnt4 induces tubulogenesis in an organ culture,[3] suggesting both essential and sufficient roles of *Wnt4* in the epithelial differentiation of the metanephric mesenchyme. More recently, *Wnt9b* expressed in the ureteric bud was shown to function upstream of *Wnt4*.[4] Thus, we attempted to set up an assay using *Wnt4*, which could identify and characterize the progenitor cells with multipotent differentiation potential from uninduced metanephric mesenchyme. We previously generated mice in which the green fluorescence protein (*GFP*) gene was knocked into the locus of *Sall1* (*Sall1-GFP* mice), a zinc-finger nuclear factor that is expressed in the metanephric mesenchyme and is essential for kidney development.[5,6] *Sall1* is also expressed in the subventricular zone of the central nervous system and progress zones of limb buds, where neural and mesenchymal stem cells reside, respectively, leading to speculation that *Sall1* might have some association with stem/progenitor cells in several organs, including the kidney. When we plated dissociated mesenchymal cells at a low density in serum-free conditions onto NIH3T3 cells stably expressing *Wnt4*, a single cell formed colonies consisting of several types of epithelial cells that exist in glomeruli and renal tubules.[7] This indicates that the single cell plated initially may be a multipotent renal progenitor, which differentiates into glomerular podocytes and the epithelial cells of renal tubules. We also found that only cells strongly expressing *Sall1*, isolated from *Sall1-GFP* mice, formed colonies and that they partially reconstituted a three-dimensional kidney structure, which contains glomeruli- and tubule-like components in an organ culture setting (Figure 1). Thus, multipotent renal progenitors of epithelial cells do exist and they reside in the *Sall1*-high population of the mesenchyme. The *Sall-GFP* high fraction constitutes 20–30% of the mesenchymal cells during embryonic development and is distinct from stromal cells and hemangioblasts, as assessed by gene expression patterns. We next generated colonies from *Sall1*-deficient mice to address the role of *Sall1* in the renal progenitors. The numbers of colonies formed were not significantly different among wild-type, heterozygous, and homozygous mesenchyme, suggesting that colony-forming progenitors do exist in the absence of *Sall1*. *Sall1*-null colonies were positive for E-cadherin and marker genes for terminally differentiated epithelia, suggesting that differentiation (including mesenchymal-to-epithelial transformation) may not be impaired in the absence of *Sall1*. By contrast, the size of *Sall1*-deficient colonies was significantly smaller than that of heterozygous or wild-type colonies. Thus, *Sall1* is not required for the generation or differentiation of renal progenitors, but it may be required for the proliferation/survival of the kidney progenitors. As *Sall4*, another member of the *Sall* family of genes, is essential for proliferation of embryonic stem (ES) cells, the *Sall* family may have a common role of stem cell/progenitor proliferation.[8] Thus, our colony-forming assay, which identifies multipotent progenitors of epithelial cells in the embryonic mouse kidney, can be used to examine molecular mechanisms functioning in kidney development. Moreover, the colony assay will possibly be useful for the identification of renal progenitors induced from a variety of cell sources, such as ES cells.

IDENTIFICATION OF GENES EXPRESSED IN PROGENITORS BY MICROARRAY ANALYSIS

In the embryonic kidney, *Sall1* is expressed abundantly in mesenchyme-derived structures from undifferentiated mesenchyme, comma-, S-shaped bodies, and renal tubules. We isolated the *GFP*-positive population from embryonic kidneys of *Sall1-GFP* mice by fluorescein-activated cell sorting (FACS) and compared the gene expression profiles of *GFP*-positive (mesenchyme) and *GFP*-negative (ureteric bud) populations using microarray analysis, followed by *in situ* hybridization.[6] We detected many genes known to be important for metanephros development, including *Sall1*, *GDNF*, and *Six2* (which will be discussed later), and genes expressed abundantly in the metanephric mesenchyme, such as *Unc4.1* and *Osr2*. Therefore, the combination of microarray technology and *Sall1-GFP* mice is useful for the systematic identification of genes expressed in the developing kidney.

Identification of surface markers expressed in the mesenchymal progenitor population is useful, as it is possible to isolate progenitors from mice that have not been genetically manipulated, and potentially also from humans. Challen *et al.*[9] performed microarray analysis using undifferentiated mesenchyme and found that *CD24* and *cadherin-11* are preferentially expressed in the mesenchyme. Therefore, these may prove useful in the purification of mesenchymal progenitors by FACS.

MAINTENANCE OF PROGENITORS BY Six2

The *Six* homeobox genes are characterized by conserved Six domains and a homeodomain, both of which are required for specific DNA binding. The prototype of this gene family is *Drosophila sine oculis*, which is essential for compound eye formation. Six members (*Six1–Six6*) of the *Six* gene family have been identified in mice and humans. Among these, *Six2* is expressed in the undifferentiated mesenchyme that caps the ureteric bud, and its expression pattern is reciprocal to that of *Wnt4*, which is expressed near the ureteric stalk. *Wnt4* and the Wnt agonist *sFRP2* are upregulated in the cap portion of the mesenchyme in *Six2*-deficient mice, and ectopic tubulogenesis is observed.[10] In addition, overexpression of *Six2* in mesenchymal cells prevents their epithelial differentiation in an organ culture assay. These results suggest that *Six2* is required to maintain the mesenchymal progenitor population by opposing *Wnt4*-mediated epithelialization. As *Six2* is expressed in *Sall1*-high mesenchymal cells, as described in the previous section, the *Sall1*-high and *Six2*-positive mesenchyme may be a progenitor population in the kidney, and *Six2* might maintain the mesenchyme in an undifferentiated state (Figure 2). It would be interesting to test whether sustained expression of *Six2* maintains the progenitors in our colony assay and also *in vivo*.

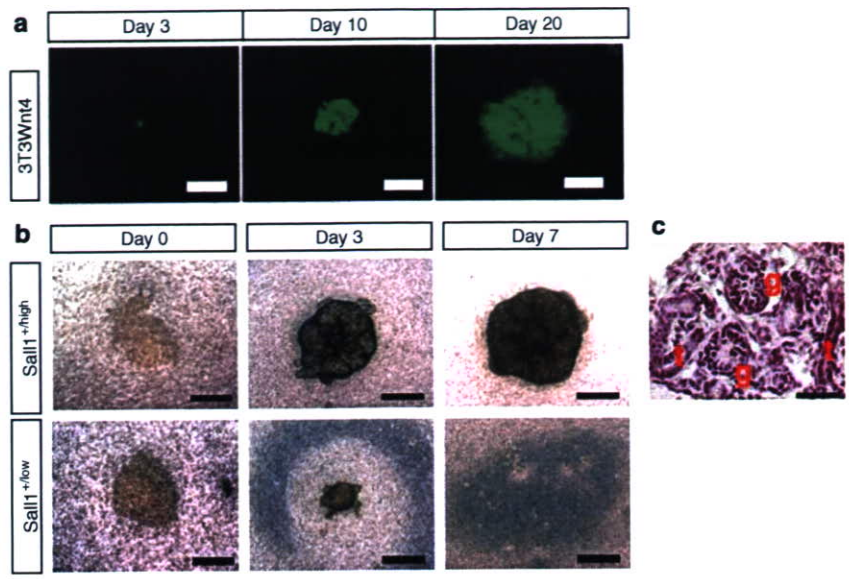

Figure 1 | Metanephric mesenchymal progenitors. (**a**) *In vitro* colony formation from the metanephric mesenchyme on feeder cells expressing Wnt4. (**b**) Only Sall1-GFP^{high} cells (upper panels) differentiated into kidney structures in organ culture, whereas Sall1-GFP^{low} cells (lower) disappeared. (**c**) Hematoxylin–eosin staining of sections of Sall1-GFP^{high} aggregates at day 10. Tubule- (t) and glomerulus-like structures (g) are seen. Bars: (**a**) 50, (**b**) 500, (**c**) 25 μm.

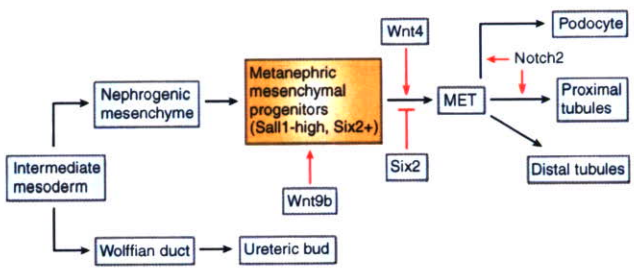

Figure 2 | Kidney development in view of stem cell biology. See the text for details. MET, mesenchymal-to-epithelial transition.

TRANSITION FROM THE PROGENITOR TO TUBULES BY WNT SIGNALING

As described above, *Wnt9b* and *Wnt4* are essential for the epithelial transformation of the mesenchymal progenitor into renal tubules. *Wnt9b* is secreted from the ureteric bud, and consequently Wnt4 is induced in the mesenchyme where it functions in an autocrine manner on the mesenchyme itself. Downstream of Wnts, β-catenin is likely to play an important role. Removal of β-catenin from *Six2*-positive progenitors blocks the epithelial conversion of the mesenchyme.[11] By contrast, activation of β-catenin in the same cell population partially replaces the requirement for *Wnt9b* and *Wnt4*. However, complete epithelial conversion of mesenchymal progenitors does not occur by β-catenin activation, suggesting that further modulation of canonical signaling will be required for the complete progression of tubulogenesis.

Microarray analysis of cultured rat mesenchyme also shows that β-catenin-dependent signaling is activated and directly regulates *cyclin D1*, *Pax8*, and *Emx2*.[12] β-Catenin activation leads to the survival and proliferation of epithelial progenitors, but not epithelial conversion, suggesting again that additional pathways downstream of Wnt signaling may cooperate with β-catenin. Indeed Rac- and c-Jun N-terminal kinase-dependent planar cell polarity pathways downstream of Wnt4 positively regulate the size of the colonies formed from the mesenchyme.[7] Nonetheless, these results enforce the importance of Wnt signaling in the epithelial conversion of the mesenchyme.

NEPHRON SPECIFICATION BY NOTCH2

The mesenchyme differentiates into epithelia after Wnt stimulation, but a fate decision is required for further differentiation toward glomerular podocytes and proximal or distal renal tubules. *Notch2* is required for the differentiation of proximal nephron structures (podocyte and proximal tubules), as mesenchyme-specific *Notch2* deletion leads to impaired formation of these proximal structures.[13] Although proximodistal polarity is initiated in the absence of *Notch2*, the latter is essential for the final establishment of proximal fates. It remains to be solved whether *Notch2* functions through lateral inhibition or other mechanisms, but elucidation of this process could lead to the cell fate manipulation of kidney progenitors, which should be a useful technology for kidney regeneration: stimulation of progenitors in the mesenchyme by Wnts followed by *Notch2* ligand may result in the generation of the necessary lineages in patients (Figure 2). Interestingly, *Notch1* cannot compensate for *Notch2* deficiency, although Notch1 activity is present in the kidney and increasing its activity enhances the formation of proximal tubules at the expense of podocytes and distal nephrons.

Of course, there are likely to be many more genes involved in lineage commitment and the differentiation of the many

types of cells that are intermingled in the kidney. It will be important to mark and sort a certain progenitor domain using a fluorescent dye inserted by genetic manipulation and to examine the cell fate and gene expression profiles of cells in this domain. Indeed, this type of analysis is being developed, for example, by the GenitoUrinary Development Molecular Anatomy Project (GUDMAP) (http://www.gudmap.org/).[14]

THE ORIGIN OF ENDOTHELIAL CELLS

Once podocytes are formed, they secrete vascular endothelial growth factor (VEGF) and attract hemangioblasts, which differentiate into glomerular endothelial cells and their pericytes, namely, mesangial cells, thus forming vascularized glomeruli.[15] Although classical interspecies grafting experiments suggest that the vasculature is of host origin, vascular endothelial progenitors could be present in the metanephric mesenchyme, because hypoxia or VEGF treatment induces vascular formation inside the mesenchyme in an organ culture setting.[16] It has also been reported that vascular endothelial cells are detected when dissociated mesenchymal cells are cultured.[17] However, this does not necessarily imply the existence of a single multipotent progenitor that differentiates into renal epithelial and vascular endothelial cells, because vascular progenitors in the heterogeneous mesenchyme may be selectively expanded. Indeed VEGF receptor (Flk1)-positive endothelial precursors are already separated from, and surround, the mesenchyme at the stage when the interaction between the mesenchyme and the ureteric bud starts (embryonic day 11.5).[18] Consistent with this observation, *Sall*-negative, but not *Sall1*-high, mesenchymal cells express endothelial markers, including Flk1 and VE-cadherin. Thus, at least *Sall1*-high progenitors are distinct from endothelial cell precursors.[7] Careful clonal analysis is needed to address the origin of endothelial cells in the developing kidney.

THE ORIGIN OF STROMAL CELLS

Stromal cells reside in the outermost layer of the kidney cortex and surround the metanephric mesenchyme. Retinoic acid signaling in this stromal cell population leads to increased expression of Ret (the glial cell line-derived neurotrophic factor (GDNF) receptor) in the ureteric bud and subsequently regulates ureteric bud branching.[19] The origin of the stroma, however, remains unknown. At least at embryonic day 11.5, only Sall1-GFP^{low} cells express a marker of stroma (*Foxd1*, also known as *BF2*), and no colonies were formed from this population upon Wnt4 stimulation, suggesting that the stroma may not be derived from the progenitors of renal epithelial cells.[7] Thus, the developing mesenchyme could contain vascular and stromal progenitors in addition to the Sall1-high epithelial progenitors that differentiate into glomerular podocytes and renal tubules.

DERIVATION OF RENAL PROGENITORS FROM ES CELLS

Now that we know that the metanephric mesenchyme contains epithelial progenitors and how they differentiate into renal epithelial cells, how can we generate the mesenchyme itself? Clues to this come from developmental biology. The kidney develops in three stages: pronephros, mesonephros, and metanephros. The nephric duct (Wolffian duct) and nephrogenic mesenchyme develop from the intermediate mesoderm, which is located between the lateral and preaxial mesoderm. The Wolffian duct and nephrogenic mesenchyme elongate caudally toward the cloaca, and the Wolffian duct converts the adjacent mesenchyme into mesonephric tubules. Metanephric mesenchyme is formed at the caudal-most region of the nephrogenic mesenchyme.

Therefore, the progenitor population in the metanephric mesenchyme could be derived from the intermediate mesenchyme, although it remains to be determined whether metanephric mesenchyme is a direct derivative of nephrogenic mesenchyme in pro/mesonephros or a distinct population recruited from the surrounding tissue.

The transcription factors Pax2 and Pax8 control the initial differentiation from intermediate mesoderm to Wolffian duct; thus, mutant mice lacking both of these genes lack pronephros formation.[20] *Osr1* is the earliest marker expressed in the undifferentiated intermediate mesenchyme. Once Wolffian ducts are formed, *Osr1* expression disappears, whereas *Pax2* remains to be expressed in the Wolffian ducts. Although *Osr1*-deficient mice show milder pro/mesonephric phenotypes than *Pax2/Pax8*-null mice, possibly due to genetic redundancy with other *Osr* gene family members, *Osr1*-null mice show an absence of metanephric mesenchyme, placing *Osr1* upstream within the metanephric gene cascade.[21] Interestingly, *Osr1* is also expressed in the undifferentiated metanephric mesenchyme, and this gene could serve as a metanephric progenitor marker, such as *Six2*.

So, will it be possible to induce the formation of intermediate mesenchyme and nephrogenic mesenchyme of pro/mesonephros from a variety of cell sources, such as embryonic stem (ES) cells, and to direct them toward becoming metanephric mesenchymal progenitors? In frogs, treatment of the animal cap, a presumptive ectoderm of fertilized embryo, with activin and retinoic acid, efficiently and selectively induces pronephric tubules *in vitro*.[22] Indeed, *Sall1* was cloned using this assay. Kim and Dressler[23] reported that embryoid bodies generated from ES cells treated with activin, retinoic acid, and Bmp7 express several markers, including *Pax2* and *Six2*. When they cultured embryoid bodies with the spinal cord, a potent source of Wnt ligands, they formed a tubule-like structure. This is an interesting observation, but it remains to be determined whether these cells are metanephric mesenchyme or earlier stages of the kidney (pronephros/intermediate mesoderm), as in the case of the frog. Purification of a candidate cell population and a reliable functional assay are needed to verify this aspect. Nonetheless, these data suggest a mechanism of kidney progenitor formation that is conserved among species. If derivation methods of metanephric mesenchyme are established, we will be closer to the generation and manipulation of multiple cell lineages in the kidney.

DISCLOSURE
The author has no conflicts of interest.

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特集 哺乳類の器官構築

腎臓形成と再生

Kidney development and induction of renal lineage

内山裕佳子・西中村隆一

哺乳類の腎臓は，発生学的に中胚葉から分化する後腎に由来する．後腎は，のちに糸球体や尿管となる後腎間葉と，集合管や尿管になる尿管芽との相互作用により分化するが，その分子機構がノックアウトマウスの解析などから明らかとなってきた．腎臓は自己修復能が低く，その機能は多種類の細胞からなる複雑な構造に依存していることから，再生の困難な臓器と考えられてきたが，臓器移植に代わる将来的な根治療法として再生医療への期待が高まるなか，腎臓構成細胞を分化誘導する試みも徐々に進んでいる．

Key words ●後腎間葉 ●腎臓前駆細胞 ●ES細胞 ●Sall1

はじめに：腎臓の構造と機能

腎臓は，尿産生による水・電解質平衡の維持，有害物質の除去に加え，レニン産生による血圧調節やエリスロポエチン産生による造血，ビタミンD活性化による骨代謝にも関与する．尿の産生と排泄の機能単位はネフロンとよばれ，片側の腎臓に約100万個のネフロンが存在する．ネフロンは，毛細血管の塊である糸球体とそれを包むボーマン嚢，それにつづく近位尿細管，遠位尿細管からなり，集合管へとつながる．おのおののネフロン由来の集合管は最終的に1本の尿管となり膀胱へ達する．ネフロンを構成する細胞は多様であり，血管内皮細胞，糸球体上皮細胞（たこ足細胞），尿細管上皮細胞，メサンギウム細胞，糸球体傍細胞など，少なくとも12種類以上の細胞が存在する（図1）．

このような腎臓の特異な構造はどのように形成されるのだろうか？ その発生過程を知ることにより，腎臓再生への足がかりが得られるだろう．近年，腎臓発生の分子機構が急速に明らかになってきており，*in vitro*での腎臓細胞再生へむけた試みも徐々に行なわれている．本稿では，前半で腎臓の発生過程について概説し，後半で腎臓の再生研究の現状と今後の展望を述べる．

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I 腎臓の発生

1. 腎臓発生の概略

腎臓は中胚葉に由来し，哺乳類では前腎，中腎を経て，最終的な腎臓である後腎が発生する（図2）．マウスでは胎生7.5日に原始線条の出現により中胚葉が確立され，神経管に隣接する中胚葉からは沿軸中胚葉（体節）が生じ，より遠位の中胚葉からは中間中胚葉と側板中胚葉が生じる．腎臓は，沿軸中胚葉と側板中胚葉とのあいだに位置する中間中胚葉に由来する．胎生8.5日に中間中胚葉から前腎管が生じ，前腎管の吻側では周囲の中胚葉から前腎が生じる．前腎は発生過程で退化するが，前腎管の尾側はウォルフ管（Wolffian duct）として残存する．前腎よりも尾側でウォルフ管から約50個の中腎が形成され，糸球体と近位尿細管様の構造を形成するが，男性生殖腺へ分化する一部を除き，ほかは前腎と同様に退化する．成体で機能する永久腎は，中腎よりもさらに尾側に形成される後腎に由来する．

後腎の発生は，胎生10.5日にウォルフ管の尾側から尿管芽（ureteric bud）が背側へむかって分枝し，間葉細胞集団へ伸長することによりはじまる（図3）．後述するように，尿管芽と後腎間葉の2つの構成要素の相互的な誘導作用により後腎が形成される．尿管芽の先端が枝分かれを重ねる一方，後腎間葉細胞は伸長した尿管芽の先端に集合して上皮化する．これは間葉-上皮転換（mesenchymal-to-epithelial transformation；MET）とよばれ，尿管芽の伸長・分岐とともに後腎形成の重要なステップである．上皮化した間葉細胞の集合体はC字体を経てS字体となる．S字体では

近位-遠位軸が成立しており，近位部に血管内皮細胞が侵入して糸球体へ分化する．遠位部は尿管芽と融合し，近位尿細管，遠位尿細管を形成する．一方，尿管芽は集合管，尿管へ分化する．そのほかの間葉細胞は間質を形成する．尿管芽は放射状に伸長と分岐を続けるので，新しいネフロンほど腎臓の辺縁近くに位置することになる．

2. 腎臓発生の分子機構

このように，最終的にネフロンを構成する細胞の大部分は後腎間葉から尿管芽との相互作用により分化し，その分子機構については詳細な解析が行なわれている．一方，中胚葉から前腎，中腎が分化する過程や，後腎間葉から最終的に多種類の細胞が分化する過程については未解明の部分が多い．

A. 中間中胚葉の運命決定

中間中胚葉の運命決定にはBmpシグナルが関与すると考えられている．アフリカツメガエルの中胚葉ではBmp4の濃度により，高濃度では血液，中濃度では腎臓，低濃度では筋や脊索が誘導される[1]．ニワトリでは，高濃度のBmp2は側板中胚葉を誘導し，低濃度では中間中胚葉を誘導することが示されている[2]．哺乳類における中胚葉分化の分子基盤についての報告はほとんどない．

B. 前腎，中腎の形成

中間中胚葉では，形態的な変化以前にOsr1，Pax2，Lim1などのマーカーが発現する．現在，知られているなかでもっとも早期に発現がみられるのはOsr1であり，胎生8.0日以降，間葉で発現が認められるが，上皮化した細胞では検出されない．Osr1ノックアウトマウスではウォルフ管や中腎の形成に異常が認められ，後腎間葉も欠損する．ニワトリの中胚葉でOsr1を異所性に発現させるとPax2などのマーカーが誘導されることから，Osr1は腎臓前駆細胞の誘導に必要であると考えられる[3]．Osr1ノックアウトマウスは後述のPax2とPax8のダブルノックアウトマウスよりも軽い表現型を示すが，アフリカツメガエルやゼブラフィッシュでOsr1とOsr2の両方をノックダウンすると腎構造が形成されないことから[4]，Osr1はOsr2によって補われている可能性もある．

一方，Pax2は胎生8.5日より腎管と間葉の両方で発現が認められる．Pax2を異所性に発現させると腎上皮に分化することから，Pax2は腎臓系列の運命決定にかかわっていると考えられる[5]．Pax2とPax8のダブルノックアウトマウスの中間中胚葉では，前腎管形成に必要な間葉-上皮転換がみられず，前腎，中腎，後腎が形成されない[5]．Pax2のみのノックアウトマウスはダブルノックアウトマウスよりも軽

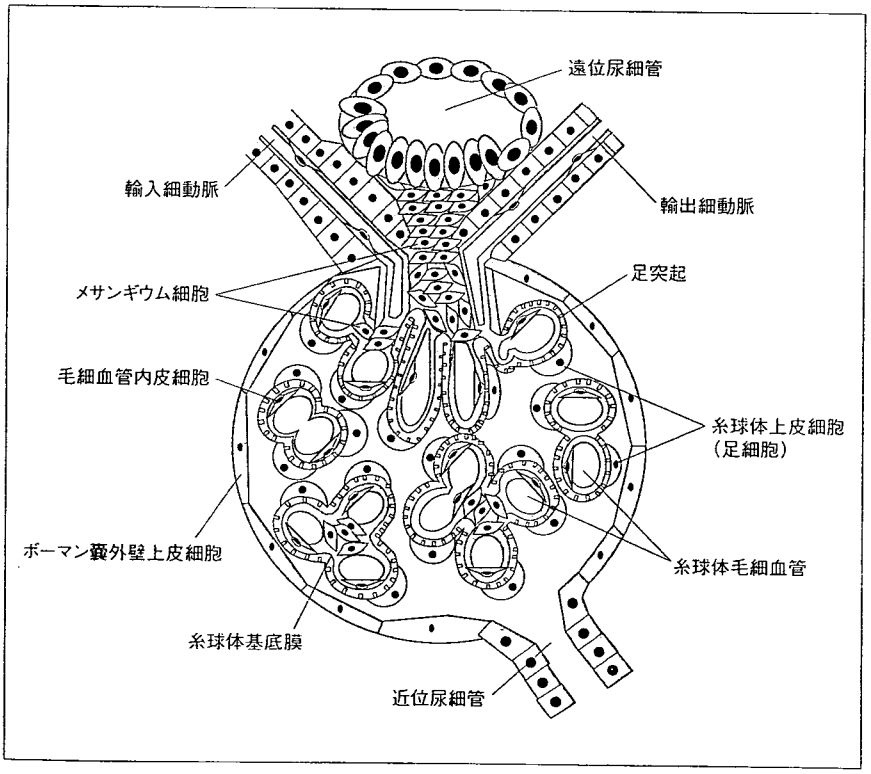

図1　糸球体の構造

糸球体は，毛細血管網とその支持組織であるメサンギウム細胞，それらを覆う内外2層の糸球体上皮細胞，および，ボーマン嚢外壁上皮細胞からなる球状の小体で，輸入細動脈および輸出細動脈と接続している．腎臓には毎分約1.2リットルの血液が流れ込み，糸球体で毛細血管内外の圧力差により濾過され原尿となる．毛細血管内皮細胞，糸球体基底膜，および，足細胞の3者が濾過膜を形成し，選択的透過を行なう．原尿は尿細管で物質の分泌や水分の再吸収を受け，尿として排泄される．尿細管の機能は，尿細管が形成する特殊なループ構造（ヘンレ係蹄）や，尿細管上皮細胞が部位特異的に発現するさまざまなチャネルやポンプに依存している．

い表現型を示すが[6]，これはPax8がPax2と重複した機能をもつためと考えられる．ニワトリにおける解析から，近接する外胚葉[7]や沿軸中胚葉（体節）[8]からのシグナルが中間中胚葉におけるPax2の発現に重要であるものと考えられている．また，Lim1ノックアウトマウスは後腎を欠損するが，中腎までは形成されPax2を発現することから，Lim1はPax2の下流で機能すると考えられる．

C．後腎形成

後腎形成は2つの重要なイベント，すなわち，尿管芽の分岐・伸長と間葉-上皮転換のくり返しにより進行する．この過程で，尿管芽と間葉のあいだには相互的なシグナル作用がみられる．間葉から分泌されるGDNF（glial cell line-derived neurotrophic factor，グリア由来神経栄養因子）は，尿管芽に発現するGDNF受容体であるRetおよびその

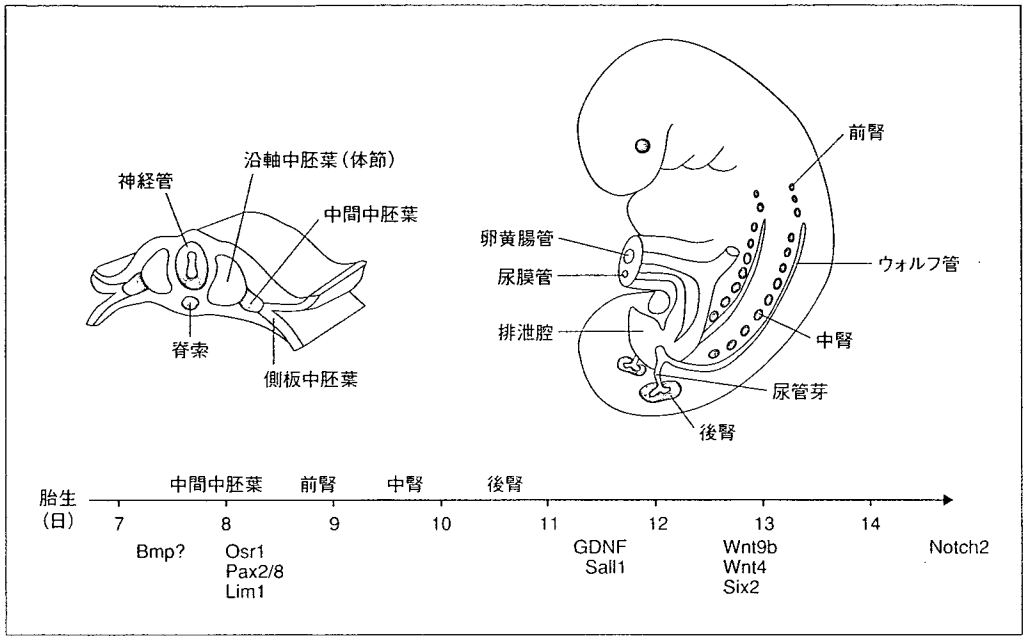

図2　マウスにおける腎臓発生の時系列

哺乳類では，中間中胚葉から3つの腎臓が吻側から尾側へむかって順次形成される．このうち，前腎，中腎は退化し，最終的には後腎が成体で機能する．下部に，腎臓形成に関与する遺伝子の一部を示す．

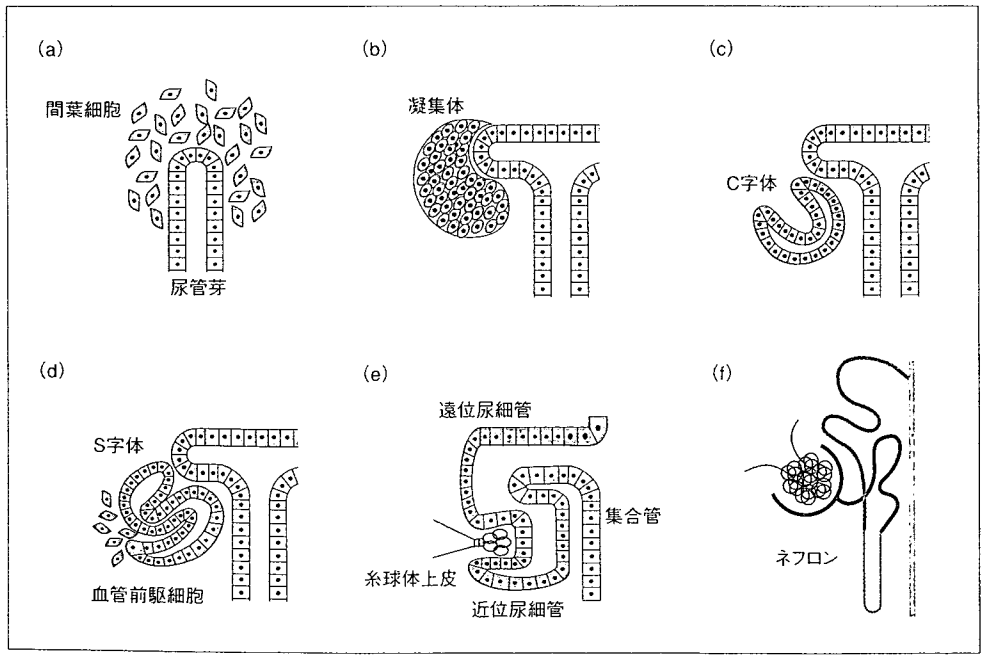

図3　ネフロンの発生

胎生10.5日にウォルフ管の尾側から尿管芽が分岐し，後腎間葉へ侵入を開始する(a)．間葉細胞は尿管芽の周囲に凝集体をつくる(b)．凝集体はC字体を経て(c)，S字体となり，血管前駆細胞を取り込みながら，遠位で尿管芽と融合する(d)．S字体の部位ごとに糸球体上皮，近位尿細管，遠位尿細管へと分化する(e)．糸球体，尿細管，集合管を合わせた腎臓機能の最小構成単位をネフロンとよぶ(f)．

共同受容体であるGDNF受容体αに作用し，尿管芽の分岐と伸長を促進する．一方，尿管芽から分泌されるWnt9bは間葉におけるWnt4の発現を促進し[9]，Wnt4は間葉自身に作用して間葉-上皮転換を促進する．これらの過程にかかわる多くの因子が知られており，詳細はほかの総説を参照されたい[10]．

D．糸球体，尿細管への分化

糸球体や尿細管の運命決定にはNotchシグナルが重要なはたらきをしている．Notchを切断して活性型にするγセクレターゼの活性を欠損したマウス腎臓ではNotch1からNotch4まですべてのNotchシグナルが阻害されるが，このマウスではC字体やS字体が形成されず糸球体と近位尿細管が欠失する[11]．別の報告によると，糸球体と近位尿細管の分化にはNotch2が必要である[12]．一方，ヘンレ係締の分化にはBrn1が必要であることが示されている[13]．

糸球体の血管内皮細胞やメサンギウム細胞は血管前駆細胞から分化する．血管前駆細胞の由来が，後腎間葉かあるいは腎臓外部かについては意見が分かれている．糸球体上皮はVEGF（vascular endotherial growth factor，血管内皮細胞成長因子）を分泌し，血管前駆細胞から内皮細胞への分化を促進する[14]．内皮細胞はPDGFβ（platelet-derived growth factor β，血小板由来成長因子β）を分泌し，血管前駆細胞からメサンギウム細胞の分化を促進する[15,16]．

II 腎臓の再生

腎臓の再生といっても，多種類の細胞からなる複雑な3次元構造を構築し，さらに血流や神経支配を適正に配置するような技術は，現時点で実現性が低い．腎臓の再生研究における当面の目標は，特定の腎臓細胞を誘導し細胞移植により損傷部位の機能を回復することであろう．移植細胞の分裂による組織再生に加え，細胞が周囲の残存組織に与える再生促進シグナルなどの効果も期待される．また，細胞移植に加えて，コラーゲンなどの生体材料を用いた足場に誘導した細胞を植えつけ，機能的な組織切片や臓器を構築して移植する組織工学の技術も進んでいる．最良の治療効果を得るためには，個々の病態にあわせて特定の分化誘導段階にある細胞を十分な量得る技術が必須である．

腎臓の再生医療に利用されうる細胞資源としては，成体前駆細胞，胎仔性前駆細胞，ES細胞があげられる．

1．成体前駆細胞

成体の腎臓にその幹細胞は存在しないと考えられてきたが，最近になって，さまざまな前駆細胞の候補が提唱されている．これまでに報告されている候補として，DNA標識物質ブロモデオキシウリジン（BrdU）の蓄積により区別される分裂速度の遅い（slow-cycling）細胞[17]，色素Hoechst 33342の排泄能が高いことで区別されるSP（side population）細胞[18]，$CD24^+CD133^+$細胞[19]，Oct4発現細胞[20]，などがあげられる．しかし，これらが幹細胞の条件である自己複製能と分化能をあわせもつかどうかの検定は，現段階では腎臓マーカー発現で確認されるのみであって，さらに機能的アッセイを行なう必要がある．また，腎臓外に存在する成体前駆細胞として骨髄間葉系幹細胞や造血幹細胞があり，骨髄間葉系幹細胞が腎臓系列の細胞に分化したとの報告もある[21]．

2．胎仔性前駆細胞

マウスでは生後10日前後までネフロン形成が続いており，少なくとも胎生期マウスの後腎間葉には，多種類の腎臓構成細胞に共通の祖先である前駆細胞が存在する．筆者らは，後腎間葉に発現する*Sall1*遺伝子を指標として，胎生期マウス後腎から腎臓前駆細胞の単離培養を行なった（図4）．*Sall1*遺伝子は腎臓発生に必須の遺伝子であり，Sall1ノックアウトマウスは尿管芽が後腎間葉へ伸長せず，後腎を欠損して出生直後に死亡する[22]．*Sall1*遺伝子座に蛍光蛋白質GFPを導入したノックインマウスの後腎間葉から，FACS（fluorescence activated cell sorting，フローサイトメーター）を用いてGFP高発現細胞を選別し，Wnt4を発現するフィーダー細胞上で培養すると，1個の細胞からコロニーが形成され，糸球体，近位尿細管，遠位尿細管のマーカーを発現する．また，これらのGFP高発現細胞を再凝集させ器官培養すると，3次元構造を再構築し糸球体や尿細管様の構造が認められる[23]．

そのほか，後腎間葉中に存在する前駆細胞の指標として，胎生10.5日から後腎間葉に発現するSix2が注目されている．Six2ノックアウトマウスでは異所的に間葉細胞の早熟な上皮化が起こり，前駆細胞が減少し腎臓が低形成となる．Six2ノックアウトマウスの後腎間葉でWnt4の発現領域が拡大していることから，Six2はWnt4シグナルを抑制することにより間葉-上皮転換を阻害し，後腎間葉の前駆細胞を未分化な状態に保っているものと考えられる[24]．

3. ES細胞

ES細胞（embryonic stem cell，胚性幹細胞）を成体に移植すると奇形腫を形成し，その組織中には糸球体様の構造も確認される．*in vitro*でES細胞から腎臓系列の細胞を誘導することができれば，容易かつ豊富に得られる細胞資源として有用である．ヒトES細胞の利用には倫理的な問題があるが，分化した皮膚線維芽細胞からの多能性幹細胞誘導が報告されており[25]，体細胞をES細胞化し，そこから目的の細胞を分化誘導する方法によって倫理的問題を回避できる可能性が出てきた．また，ES細胞から*in vitro*で腎臓細胞を誘導・単離することができれば，さまざまな分化誘導段階にある細胞を多量に得ることができるため，正常発生過程のモデルとして，また，疾患の研究材料や薬効評価の道具としても利用価値が高い．

ES細胞からの腎臓細胞誘導については，これまでにいくつかの報告がある．Wnt4を導入したマウスES細胞から胚様体を形成させ，HGF（hepatocyte growth factor，肝細胞増殖因子）とレチノイン酸を添加すると，尿細管の分化が確認される[26]．別の報告では，マウスES細胞から胚様体を形成させると糸球体様の構造を形成し，足細胞のマーカーであるネフリンや遠位尿細管マーカーであるTHPなどを発現する[27]．また，アフリカツメガエルでは，胞胚期の予定外胚葉領域（アニマルキャップ）をアクチビンとレチノイン酸で処理すると前腎管が誘導される[28]．前述の*Sall1*遺伝子もこの系を利用してクローニングしたものである．アフリカツメガエルでの結果にヒントを得て，マウスES細胞からアクチビン，レチノイン酸およびBmp7存在下で胚様体を形成させ脊索と共培養すると，中間中胚葉のマーカーを発現し尿細管構造が確認されるとの報告もある[29]．

このように，腎臓前駆細胞を単離するさまざまなアプローチが行なわれているが，これまでの腎臓再生研究では，前駆細胞を確実に同定するアッセイ系が欠けていた．これに対し，筆者らの確立したSall1-GFPノックインマウスの後腎間葉から前駆細胞を単離培養する系は，前駆細胞を1個の細胞単位で評価できる．この系に基づき，さらに，筆者らはES細胞からの腎臓前駆細胞の誘導と単離をめざしている．この際，必要十分な腎臓誘導因子を絞り込むと同時に，*in vitro*でも正常発生と同じ段階的な誘導が起こるのかを検証する必要がある．

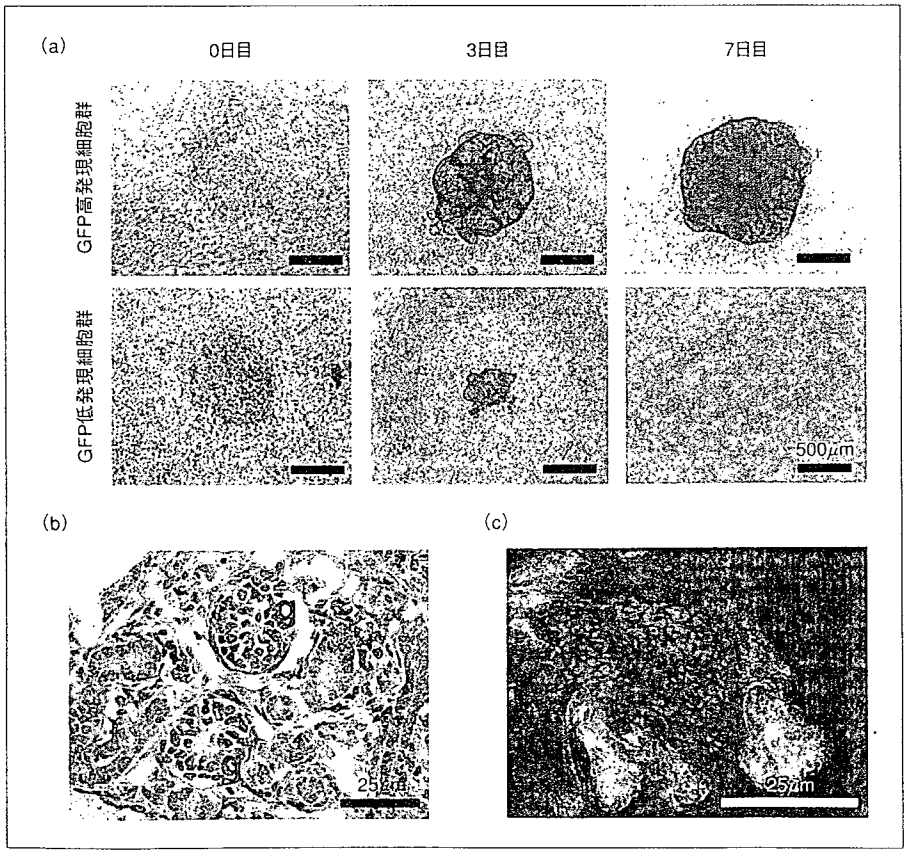

図4　後腎間葉細胞からの腎臓前駆細胞の単離

(a) *Sall1*遺伝子座にGFPを導入したノックインマウスの後腎間葉から，FACSを用いてGFP高発現細胞群とGFP低発現細胞群を選別し，Wnt4を発現するフィーダー細胞上で器官培養すると，GFP高発現細胞だけが分化して3次元構造を再構築する．

(b) 培養10日目のSall1-GFP高発現細胞群のヘマトキシリン-エオシン染色像．尿細管や糸球体様構造がみられる．g：糸球体様構造，t：尿細管様構造．

(c) Sall1-GFP高発現細胞群のWT1（赤，足細胞マーカー）とLTL（緑，近位尿細管マーカー）による二重染色像．

前駆細胞から各系列の細胞をどのように誘導するかも今後の大きな課題である．たとえば，Notchシグナルの制御により糸球体上皮や尿細管を分化誘導できるのか？　神経発生や造血では，幹細胞から数系統の前駆細胞が分化し，さらにそれぞれの前駆細胞から多様な細胞が分化する細胞系譜の概念が確立されているが，腎臓構成細胞についてはそのような系譜はいまのところ明らかでない．最終的に20種類以上もの腎臓構成細胞を生みだすおのおのの系譜を追跡するためには，*in vitro*分化系を用いたさまざまな条件下での検討が不可欠である．

おわりに

現在，わが国で人工透析を受ける人の数は26万人に達しており，腎不全などの医療費は年間1兆円をこえる．現時点で末期慢性腎不全に対する根治療法は腎移植しかないが，ドナーが決定的に不足しており，腎臓売買や病気腎移植が重大な社会問題となっている．臓器移植に代わる新しい治療として再生医療が期待されており，今後，腎臓前駆細胞，さらに，前駆細胞から糸球体，近位尿細管，遠位尿細管細胞への分化機構が解明されることで，目的の腎臓細胞を分化誘導するための手がかりが得られることを願っている．

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研究テーマ：腎臓発生．

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研究テーマ：腎臓発生をノックアウトマウスを使ってかつ幹細胞学的視点から解析している．複雑な3次元立体構造の構築や血管の配置など解明すべき点も多く，挑戦しがいのある課題だと考えており，この独創的な分野に多くの人が参入することを願っている．

【腎臓は再構築できるか？】

How to make the kidney

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Key words
metanephros, embryonic stem cell, mesenchymol stem cell, kidney development

要　約

再生医療の一環として腎臓を再構築することは現時点では困難な課題である。しかし腎臓再生に向けて，成体あるいは胎児腎臓細胞，骨髄由来間葉系幹細胞，胚性幹細胞などを使った多くの試みがなされており，尿細管や胎児腎臓の前駆細胞は同定されつつある。また間葉と尿管芽の相互作用による腎臓発生の分子機構も急速に解明が進んでいるが，さらに腎臓前駆細胞の確実な検出系の確立，前駆細胞から各種細胞系譜への運命決定機構の解析が必要である。

これらの知見を組み合わせることで，複雑な構造を有する腎臓を再構築することが必ずできるようになるはずである。

はじめに

末期腎不全のように腎機能と構造が荒廃してしまった場合に，腎臓を新たに再構築することは夢物語だろうか？多種の細胞を誘導したのちそれらを3次元立体構造に構築し，さらに血管系と結合する必要がある。これを実現するのは現時点では確かに困難である。

まず足細胞など一系統の細胞に限って誘導し，その細胞種の機能を代償することを目指すのが現実的と思われるが，これすら実現していない。しかし腎臓発生の過程をなぞることで，腎臓再構築へのヒントが得られるのではないだろうか。

本稿では腎臓発生の概略を述べたあと，現時点での再生への試みをまとめ，さらに発生に沿った腎臓再構築の可能性を考察する。

1.　腎臓の発生

腎臓は中間中胚葉から発生し，前腎，中腎，後腎の3段階を経て形成される（図1）。前腎，中腎のほとんどは後に退行変性し，哺乳類成体において機能する腎臓は後腎である。

この後腎の形成は尿管芽と後腎間葉との相互作用から始まる。後腎間葉はGDNF（glial-cell-line-derived neurotrophic factor）という液性因子を分泌して尿管芽を引き寄せる。後腎間葉に侵入した尿管芽はWnt9bを分泌し，それに反応した間葉は自らWnt4を分泌し，これが間葉自身に働いて，間葉は上皮性の管へと分化する。この管はその後S字型に変化し，その上部が近位尿細管，ヘンレのループ，遠位尿細管となり尿管芽と合流する。S字体の下部はボウマン嚢および糸球体上皮細胞(足細胞，podocyte)へと分化し，そこに毛細血管が入り込んで糸球体が形成される（図2）。

一方，尿管芽は分岐を重ね，集合管と尿管になる。この分化プロセスが分岐した尿管芽の枝一つ一つで行われ，最終的にヒトでは50万-100万個のネフロンが形成される。後腎間葉から糸球体，近位及び遠位尿細管，ヘンレのループという腎臓としての機能を司る大部分が発生することになるため，後腎間葉は多能性をもった前駆細胞集団ともいえる。

足細胞は後腎間葉由来の組織だが，毛細血管とメサンギウム細胞は血管前駆細胞由来の組織である。足細胞がVEGF（vascular endothelial growth factor）

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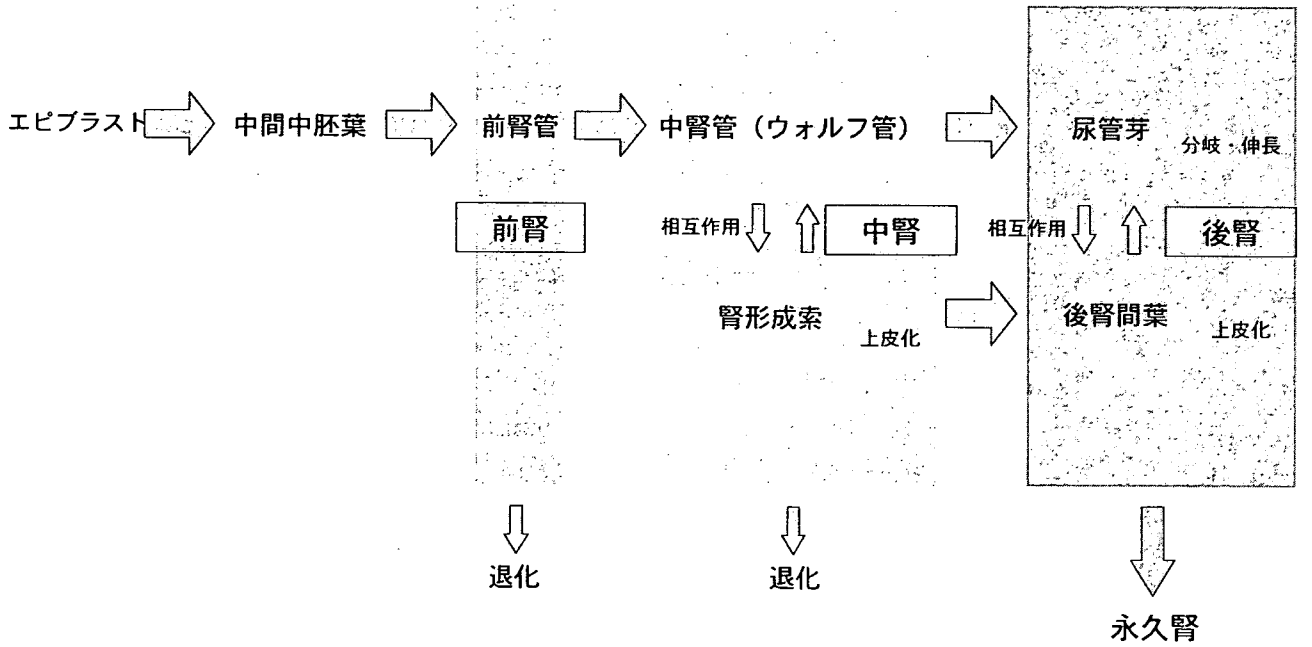

図1　哺乳類における腎臓発生

腎臓は前腎，中腎，後腎の順で形成される。哺乳類の永久腎である後腎は腎形成索の後端が特異化した「後腎間葉」と中腎管より発芽した上皮である「尿管芽」との相互作用により形成される。

を発現して血管内皮を呼び込み，さらに血管内皮がPDGF-B（platelet-derived growth factor, B polypeptide）を分泌してメサンギウム細胞を呼び込むという図式が提唱されている。

2. 腎臓再生の試み

(1) 成体腎臓内の前駆細胞

side population（SP）細胞はDNA結合色素を強く排出する性質で定義された細胞群で，骨髄中では造血幹細胞の分画を含む。よって他の臓器でもSP細胞に体性幹細胞が存在する可能性があると考えられた。しかし腎において，SP細胞が成体腎の幹細胞であると考えられる報告はない。

腎臓のSP細胞を急性腎不全モデルマウスに静脈注射すると，腎機能の改善が見られたとの報告があるが，投与されたSP細胞は間質のみに留まっており，肝細胞増殖因子（HGF）などの増殖因子を分泌することが主因であると考えられる[1]。造血幹細胞の表面抗原であるSca-1を発現する細胞が成体マウス腎臓の間質に存在し，これを分離して虚血再潅流モデルに投与したところ，尿細管や間質に存在していたとするという報告もある[2]。しかしドナー細胞とホスト細胞との融合，つまり4倍体になっている可能性を否定できていない。実際，造血幹細胞の多彩な分化能の報告のほとんどは，細胞融合によると考えられるようになっており，腎臓研究においても慎重な検討が必要である。

組織幹細胞の持つ特徴の一つに細胞周期が遅いことがある。このために幹細胞は，BrdUなどのDNA合成期に取り込まれるラベルを長く保持することになる。近位尿細管にそのような細胞が存在しており，単離してFGFやHGFの存在下で培養すると管腔構造を形成することが報告された[3]。また，近位尿細管のS3セグメントから細胞を培養し，虚血再潅流モデルに投与したところ，尿細管に成着したとの報告もある[4]。これらの細胞は，急性腎不全からの尿細管の再生時に重要な機能を果たしている可能性が示唆されるが，分化能はあくまでも尿細管にとどまり，幹細胞とはいえない。しかも急性尿細管障害は臨床的に治癒しうる病態である。

(2) 骨髄由来間葉系幹細胞

骨髄の間葉系幹細胞は患者本人から採取することが可能であり，移植しても拒絶反応の心配もない。

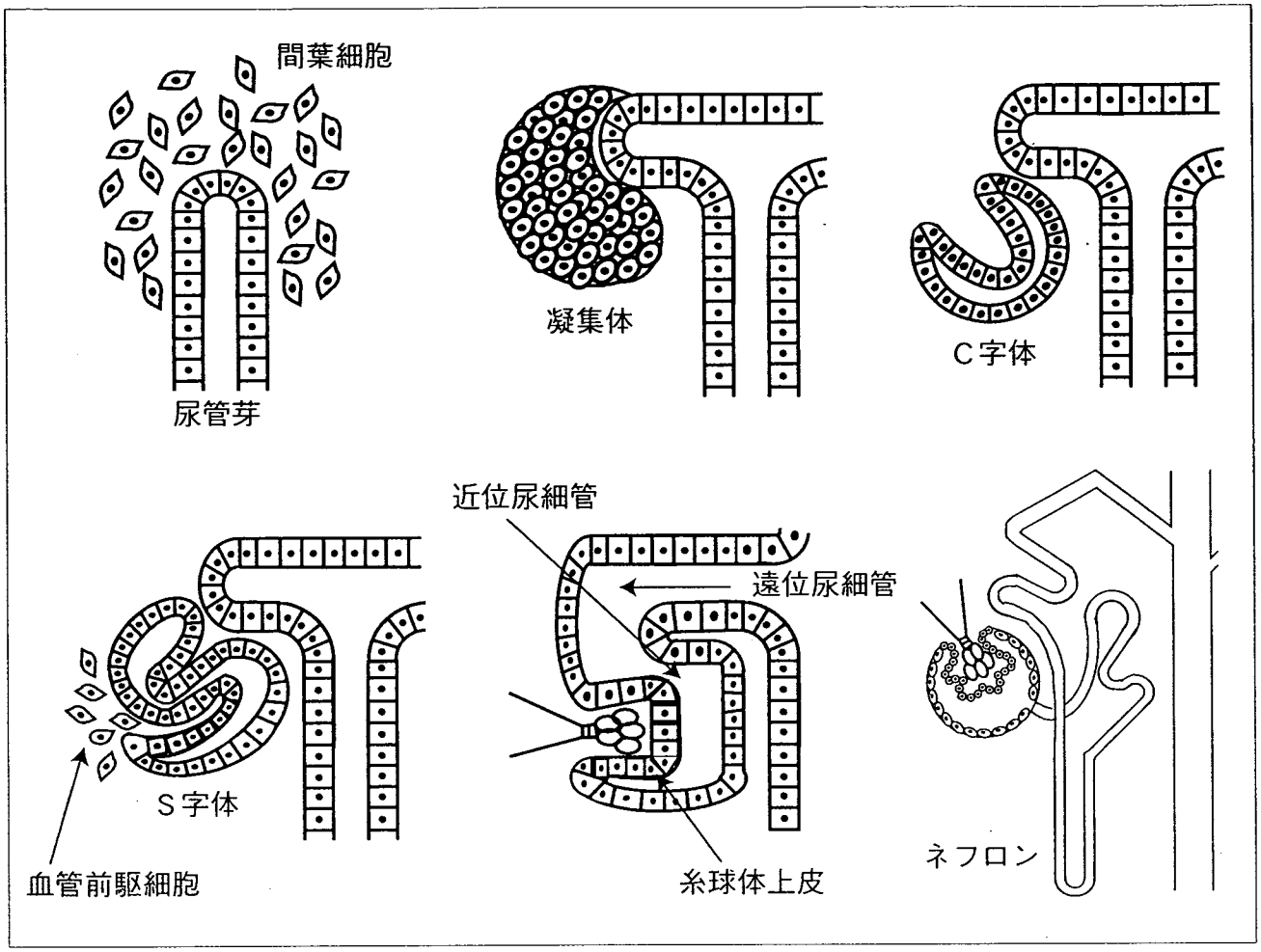

図2　後腎の発生

尿管芽の先端に後腎間葉細胞が凝集し，相互作用が起こる。尿管芽はさらに分岐し，集合管及び尿管を形成する。一方，間葉細胞は上皮化しS字体を形成する。その上部は近位及び遠位尿細管やヘンレのループを形成し，下部はボウマン嚢および糸球体上皮細胞に分化する。下部にはさらに血管が侵入し糸球体が形成される。

GDNF遺伝子を導入したヒト間葉系幹細胞を，ラット胎仔の後腎形成部位に注入し，全胚培養後その部位を摘出し，ラット大網に移植すると血管が入った腎臓の構造が作られ，このとき間葉系幹細胞由来細胞は足細胞や尿細管上皮細胞に分化していた[5]。

胎生期後腎への移植が必要なことを，臨床応用するときにどうするかは課題であるが，間葉系幹細胞の能力が示されたと言える。但しこれも細胞融合の可能性は否定されていない。

(3) 胚性幹細胞（ES細胞）

ES細胞は理論的には，体を構成するすべての細胞になりうる能力を持つ。ES細胞を浮遊培養すると胚様体を形成する。この際，レチノイン酸，アクチビンA，およびBMP7を添加するとGdnfなど発生期遺伝子の発現が見られ，これをマウス胎仔腎被膜下に注入すると，尿細管の一部にES細胞由来の細胞が成着していた[6]。またWnt4遺伝子を導入したES細胞を用いて同様に胚様体を誘導し，HGF，アクチビンAで培養すると，集合管のマーカーであるアクアポリン２の発現が見られた[7]。この他，腎臓発生で最も早期から発現するPax2にGFP遺伝子やLacZ遺伝子をノックインしたES細胞を用いて，GFPやLacZによる発色を目印にしたBMPによる誘導実験も報告されている[8]。これらも移植の際の細胞融合の検討はなされていない。

3.　腎臓の再構築を目指して

このように腎臓の再生をめざして，腎臓前駆細胞の誘導をめざす研究が多数行われている。しかし，